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14. ABSTRACT

Our results thus far support the hypothesis that the BRCAness profile is able to track diverse molecular mechanisms that cause defective homologous recombination (HR) and that it is associated with survival in patients with sporadic disease and clinical responsiveness to platinum. Specifically, application of the BRCAness profile in the TCGA EOC dataset can identify tumors that have defective HR due to overexpression of certain miRNAs, in the absence of known genetic and epigenetic abnormalities of the HR pathway. Furthermore, the HSP90 inhibitor 17-AAG enhances sensitivity of non-BRCA1/2 mutated ovarian cancer cells to DSB-inducing agents olaparib and carboplatin at very low, sublethal concentrations. Importantly, the mechanism for this synergistic effect seems to be increasing DNA damage via suppressing HR. Finally, high quality RNA from formalin fixed paraffin embedded ovarian cancer sections can be extracted and its detection and quantitation is highly concordant with that obtained from frozen tissue.

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INTRODUCTION

Patients with hereditary ovarian cancer associated with germline BRCA-1 or BRCA-2 mutations exhibit heightened sensitivity to platinum analogues and PARP inhibitors and improved survival compared to their sporadic counterparts. These characteristics are thought to be related to an underlying defect in homologous recombination that is present in these tumors. Importantly, certain sporadic tumors may also have abnormalities in HR (in the absence of germline BRCA mutations) and thus behave similarly to those with BRCA germline mutations. Such sporadic tumors are referred to as having a "BRCAness" phenotype that is characterized by heightened sensitivity to chemotherapy (platinum and PARP inhibitors) and improved overall survival. Prospective identification of sporadic tumors with a BRCAness phenotype is clinically important not only because of the advent of PARP inhibitors which exhibit synthetic lethality in tumors with defective HR pathway but also because several lines of evidence indicate that these patients may need to be managed differently.

We have developed a 60-gene expression profile that may identify tumors with a BRCAness phenotype. This profile designates tumors as BRCA-like (BL) or non-BRCA-like (NBL) corresponding to tumors predicted to have a BRCAness phenotype (BL tumors) or not (NBL tumors). In the previous years we performed a clinical validation of this profile by associating it with responsiveness to chemotherapy and outcome in epithelial ovarian cancer (Konstantinopoulos et al. Journal of Clinical Oncology;28:3555-61) (1). Furthermore, we showed that the BRCAness profile is able to track diverse molecular mechanisms that underlie defective homologous recombination (HR) in ovarian cancer including epigenetic hypermethylation of the BRCA-1 gene promoter, somatic mutations of BRCA1 or BRCA2 and loss of function mutations or epigenetic inactivation of other HR genes (Tchekmedyian et al. 2012 AACR Meeting, Chicago IL) (2). Finally, we have shown that the Keap1-Nrf2 pathway is associated with platinum resistance in ovarian cancer (Konstantinopoulos et al. Cancer Research; 71(15): 5081-9) (3) and that tumors with BRCAness (BL) profile without activation of this pathway were associated with superior outcome and responsiveness to platinum.

BODY

Aim 1: Determine whether the BRCAness gene expression profile is capable of prospectively identifying sporadic patients whose tumors exhibit defects in homologous recombination and increased sensitivity to platinum and PARP inhibitors in vitro **(months 1-60)**

A new class of gene expression regulators, microRNA (miRNA)s may down-modulate DNA repair proteins (such as BRCA1 and H2AX) in cancer cells, and impact their response to radiation, platinum and PARPi. We integrated miRNA Agilent array expression data from tumors in the TCGA EOC project that have been characterized as BL or NBL by our profile to identify 3 candidate miRNAs (let-7f-2*, miR-744*, miR-342-5p) that may be associated with defective HR in tumors that did not harbor genetic or epigenetic alterations of HR pathway genes.

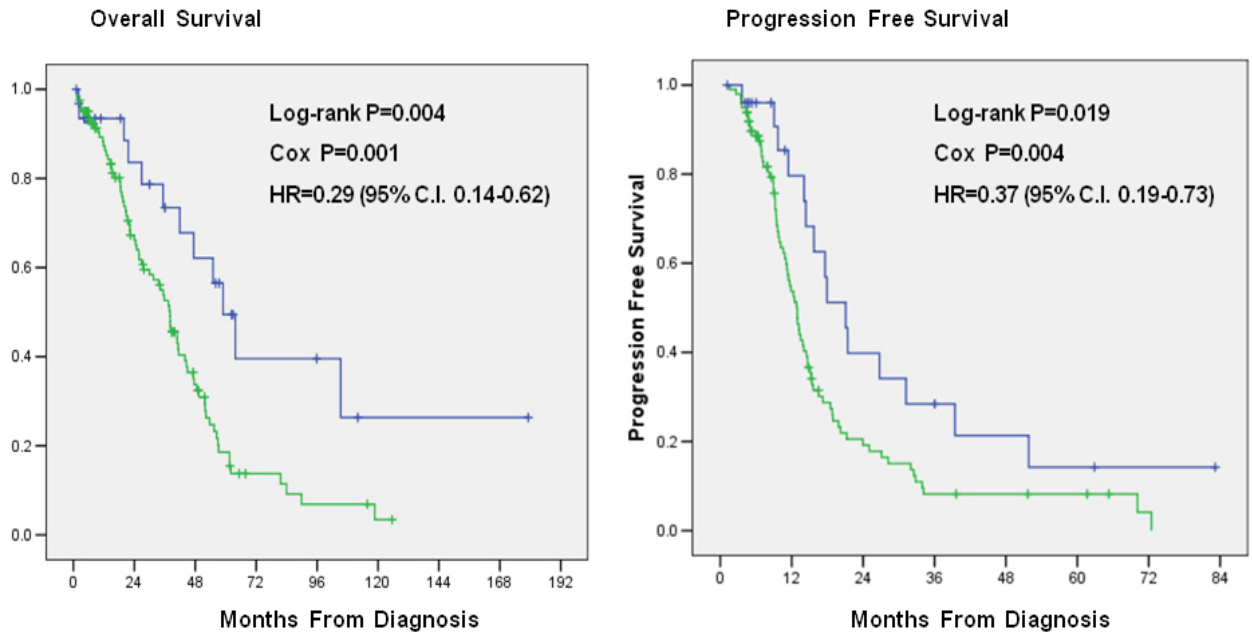

Figure 1. Tumors whose expression levels for at least one of the 3 miRNAs was in the top quintile were associated with improved OS and PFS.

This analysis identified 3 candidate miRNAs (let-7f-2*, miR-744*, miR-342-5p) that enhanced sensitivity to platinum and PARPis in vitro and were associated with improved outcome and enhanced platinum sensitivity. Among the 162 tumors without known genetic or epigenetic alterations of HR pathway genes, those tumors whose expression levels for at least one of these 3 miRNAs were in the top quintile, were associated with improved overall (OS) and progression

free survival (PFS) (Figure 1), both in univariate and multivariate analysis (Hazard ratio (HR) for death was 0.29 and for progression was 0.37).

Furthermore, among the 162 tumors without known genetic or epigenetic alterations of HR pathway genes, those tumors whose expression levels for at least one of these 3 miRNAs were in the top quintile were more likely to be platinum sensitive (i.e. developed progressive disease at least 6 or 12 months after completion of platinum based chemotherapy, Figure 3).

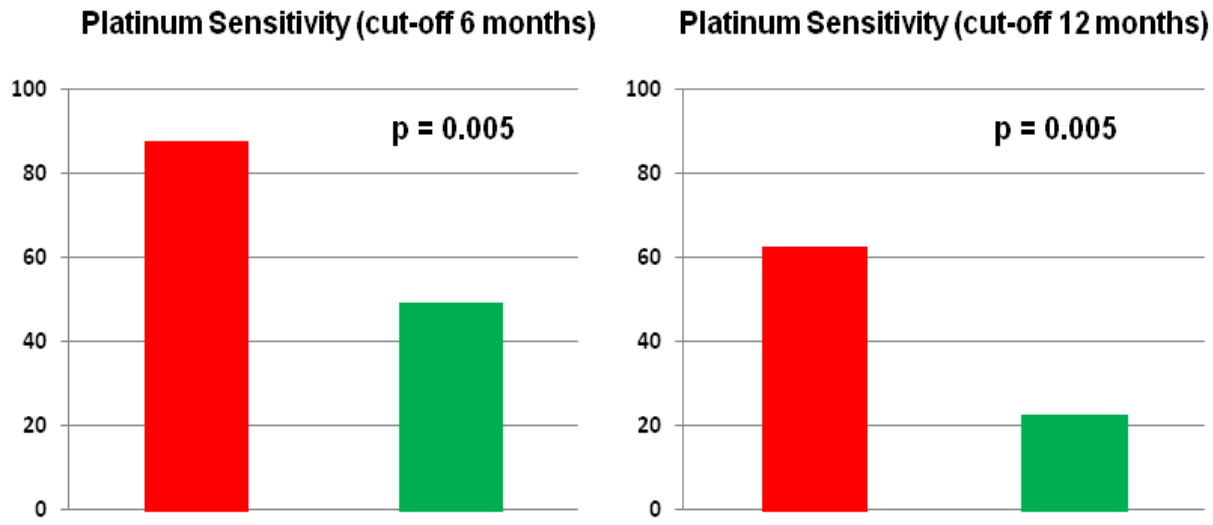

Figure 2. Tumors whose expression levels for at least one of the 3 miRNAs was in the top quintile were more likely to be platinum sensitive.

We also evaluated whether let-7f-2*, miR-744* and miR-342-5p are expressed in a panel of ovarian cancer cell lines by quantitative real time (qRT)-PCR using TaqMan MicroRNA Assay from Applied Biosystems (Figure 3). Human immortalized non-tumorigenic ovarian surface epithelial (HIO-80) cells were used as control. As shown in Figure 4, let-7f-2*, miR-744* and miR-342-5p are expressed in ovarian cancer cells and exhibit variable expression levels among different ovarian cancer cell lines.

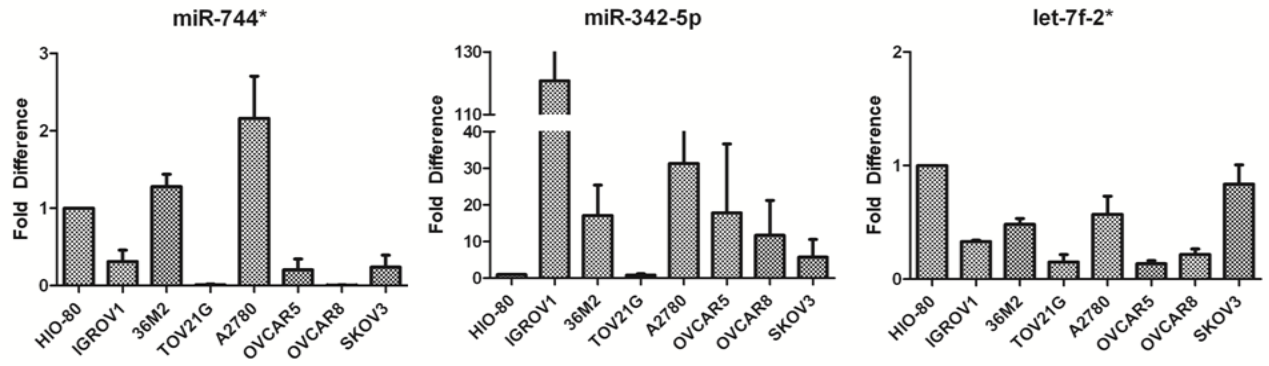

Figure 3. Expression of let-7f-2*, miR-744*, miR-342-5p in a panel of ovarian cancer cell lines (data are shown as mean ± S.D of 3 independent experiments). Human immortalized non-tumorigenic ovarian surface epithelial (HIO-80) cells were used as control.

Importantly, in vitro induced expression of miR-367* in the 36M2 ovarian cancer cell line enhances sensitivity to the DNA double strand break-inducing agent carboplatin and the PARPi olaparib (Figure 4).

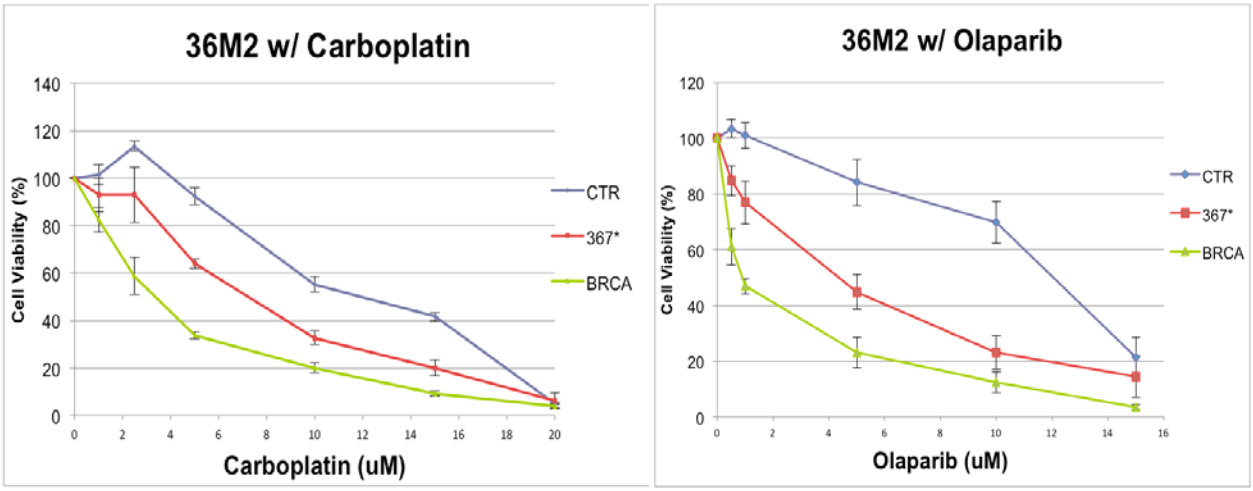

Figure 4. In vitro induced expression of miR-367* in the 36M2 ovarian cancer cell line enhances sensitivity to carboplatin and olaparib.

These data show that application of the BRCAness profile in the TCGA EOC dataset can identify tumors that have defective HR due to overexpression of certain miRNAs, in the absence of known genetic and epigenetic abnormalities of the HR pathway. This serves as a further validation that our profile is tracking sporadic tumors that harbor defective HR.

Aim 3. Evaluate whether the compounds identified by the Connectivity Map can reverse PARP inhibitor resistance in vitro, and to investigate the mechanism for this effect **(months 1-30)**

The Connectivity Map (4) has been successfully used to identify compounds that can reverse dexamethasone resistance in acute lymphoblastic leukemia. Given that our BRCAness profile may distinguish between PARP inhibitor sensitive and resistant cell lines, we applied this profile to the Connectivity Map to identify candidate compounds that might be able to reverse PARP inhibitor resistance.

A.

Cmap Name	Enrichment	P	Specificity
Geldanamycin	0.706	0	0.0163
Alvespimycin	0.63	8E-05	0.0115
Chloropyrazine	0.887	0.0002	0
Meclofenamic acid	0.83	0.0003	0
Fludroxycortide	-0.81	0.0006	0

B.

Rank	Cmap name	Dose	Cell	Score
1	Geldanamycin	1 µM	HL60	1
95	Geldanamycin	1 µM	HL60	0.792
328	Geldanamycin	1 µM	MCF7	0.679
342	Geldanamycin	1 µM	PC3	0.673
478	Geldanamycin	1 µM	MCF7	0.64
560	Geldanamycin	1 µM	MCF7	0.621
562	Geldanamycin	1 µM	MCF7	0.62
1006	Geldanamycin	1 µM	HL60	0.53
1074	Geldanamycin	1 µM	PC3	0.516

Rank	Cmap name	Dose	Cell	Score
4	Alvespimycin	100 nM	HL60	0.963
18	Alvespimycin	100 nM	HL60	0.906
116	Alvespimycin	100 nM	HL60	0.772
442	Alvespimycin	100 nM	MCF7	0.649
580	Alvespimycin	100 nM	MCF7	0.616
1104	Alvespimycin	100 nM	MCF7	0.509
1174	Alvespimycin	100 nM	PC3	0.492

Figure 5. Application of BRCAness signature into Connectivity Map identifies HSP90 inhibitors as potential compounds that could overcome PARPi resistance

Application of the top performing genes included in the BRCAness signature identified a number of interesting compounds (Figure 5A). Of note, the two highest performing compounds, geldanamycin and alvespimycin, belong to the class of HSP90 inhibitors (HSP90is) thus raising the possibility of a functional relationship between HSP90 inhibition, deficient HR and reversal of PARPi resistance. In this regard, it is predicted that these agents might reverse PARPi resistance by suppressing HR (either directly or indirectly) thereby suggesting that HSP90is may have an off-target effect involving the HR pathway. Importantly, these results were consistent even if different cut-offs for the top performing genes were chosen. As shown in Figure 5B, both HSP90is geldanamycin and alvespimycin exhibited high connectivity scores across several cell lines included in the Connectivity Map.

We then evaluated the ability of a geldanamycin analogue 17-AAG (17-allylamino-17-demethoxygeldanamycin) to enhance sensitivity to the PARPi olaparib in a panel of non-BRCA1/2 mutated ovarian cancer cell lines. We used 17-AAG, an HSP90 inhibitor that has been evaluated in clinical trials in various malignancies and has a favorable toxicity profile compared to geldanamycin which is hepatotoxic and not appropriate for clinical use.

Figure 6A shows the dose response relationship of 17-AAG as single agent in 3 of the ovarian cancer cell lines (OVCAR8, OVCAR5 and 36M2) tested. We used only sublethal doses of 17-AAG to assess whether they induce sensitivity to olaparib in these cell lines. As shown in Figure 6B, very small, sublethal concentrations of 17-AAG were associated with increased sensitivity of OVCAR8, OVCAR5 and 36M2 cells to olaparib.

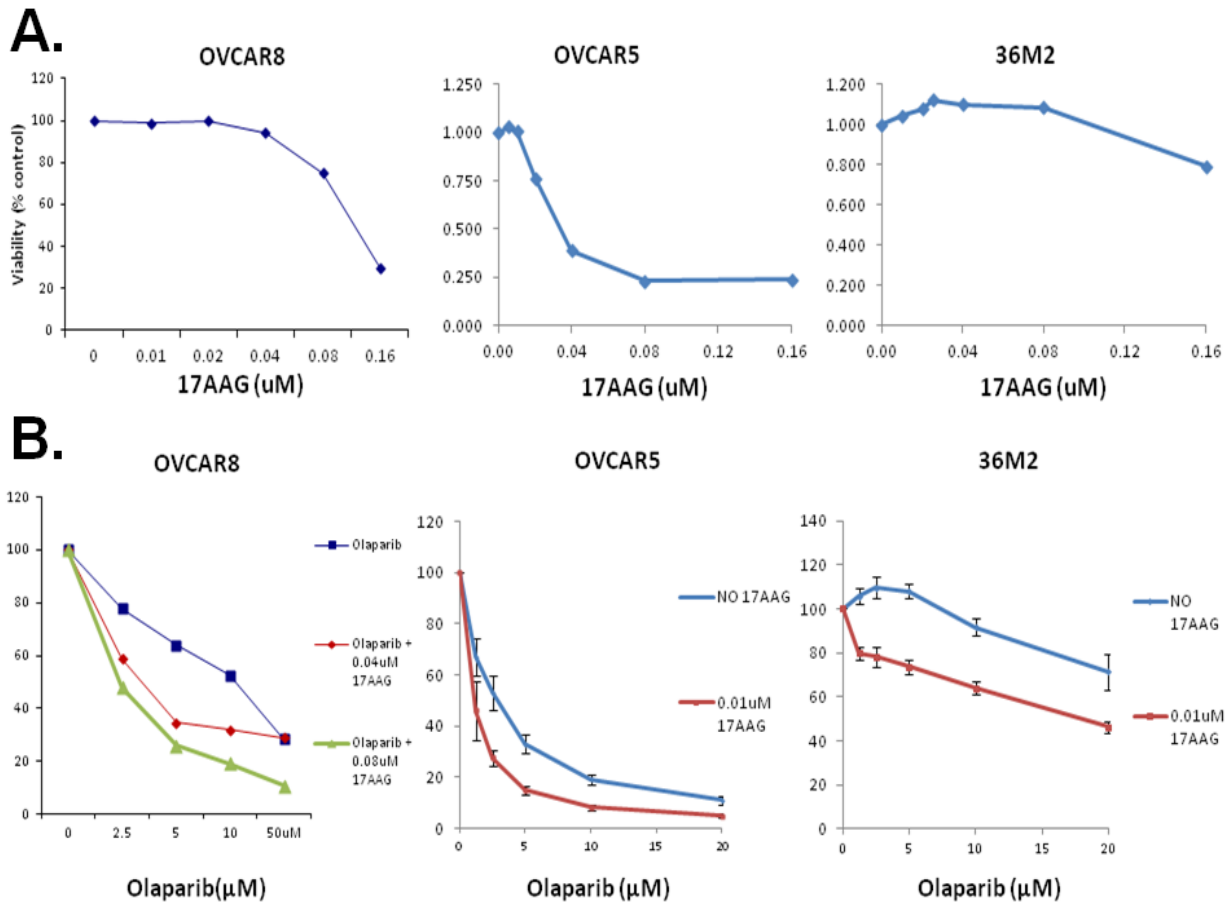

Figure 6. Exposure to sublethal concentrations of 17-AAG is associated with increased sensitivity to olaparib in non-BRCA mutated cell lines

Of note, exposure of ovarian cancer cell lines to sublethal concentrations of 17-AAG also enhanced sensitivity to other double-strand break inducing agents such as carboplatin which are repaired via HR (Figure 7A, 7B below). Furthermore, small, sublethal concentrations of 17-AAG in combination with carboplatin induced increased phosphorylation of H2AX (gamma H2AX) - a surrogate of DNA double-strand breaks - compared to carboplatin alone (Figure 3C) suggesting that 17-AAG may indeed disrupt DNA repair via HR.

In conclusion, the HSP90i 17-AAG enhances sensitivity of non-BRCA1/2 mutated ovarian cancer cells to DSB-inducing agents olaparib and carboplatin at very low, sublethal concentrations, likely via suppressing HR. It is our hope that the results of these experiments will support initiation of a clinical trial of olaparib and 17-AAG in patients with de novo or acquired resistance to PARPis.

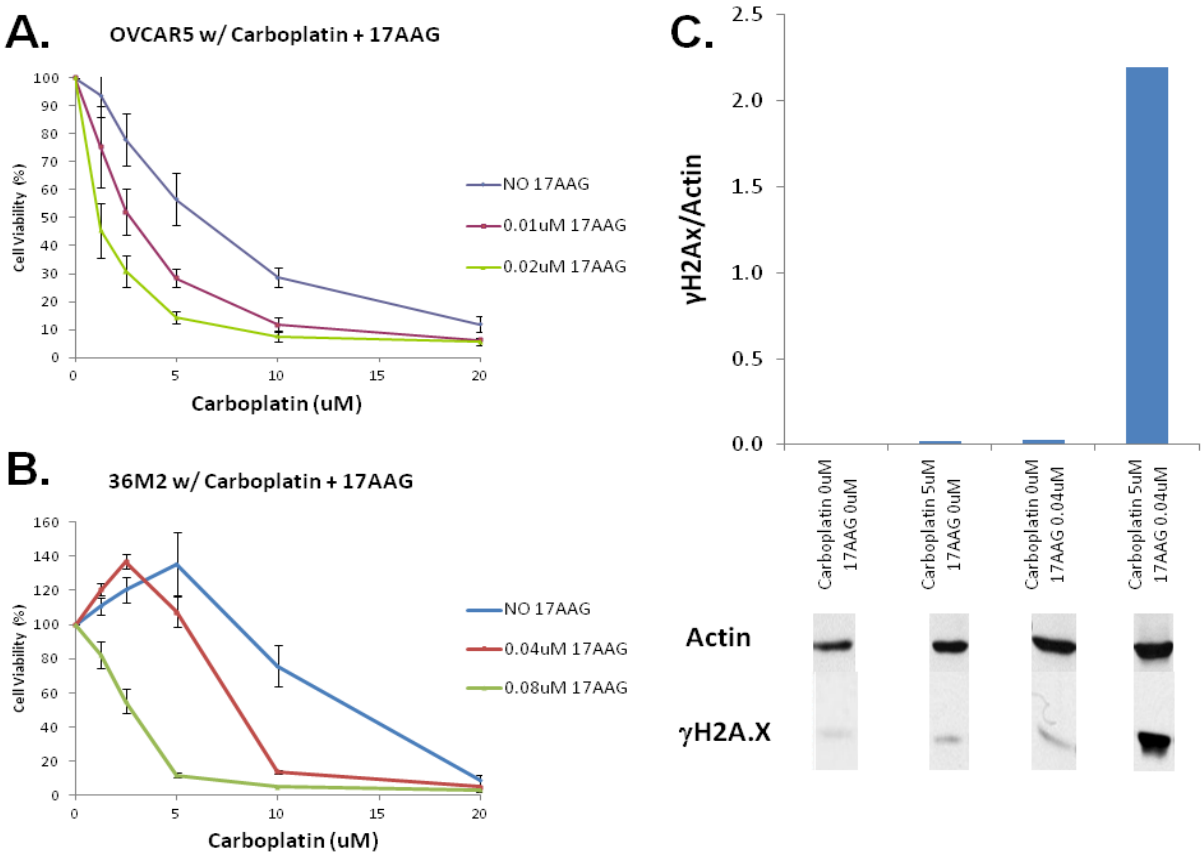

Figure 7. Exposure to sublethal concentrations of 17-AAG is associated with increased sensitivity to carboplatin and increased gH2AX levels compared to carboplatin alone in OVCAR5 and 36M2 cells

Aim 4. Determine the reproducibility of the BRCAness profile when using the DASL mRNA assay in a cohort of FFPE ovarian cancer specimens with known clinical outcome and platinum responsiveness **(months 1-42)**

We have now isolated RNA from formalin fixed paraffin-embedded (FFPE) specimens of 70 sporadic patients treated at BIDMC with known outcome and platinum response data. FFPE blocks were analyzed to identify regions containing relevant tumor cells and four to six 1 mm cores were taken from those regions using a microtome available at Beth Israel Deaconess Medical Center Histology Core facility. Cores were placed in Eppendorf tubes and paraffin was removed by sequential treatment with Xylene and ethanol. Proteins were digested with Proteinase K and total RNA extraction was performed using the Qiagen RNeasy FFPE protocol according the manufacturer's protocol. RNA quantity was measured using the Nanodrop spectrophotometer and quality was assessed using the Agilent Bioanalyzer 2100. We tested several protocols for RNA extraction but the Qiagen RNeasy FFPE protocol performed extremely well in all of our quality checks. In this regard, we used quantitative RT-PCR to measure representation of various genes to assess the level of intact message present. Further analysis of the quality of the RNA was provided by repeat assays of separate preparations from the same block.

RNA was reverse transcribed into complementary DNA (cDNA) with Promega's Reverse Transcription System according to the manufacturer's protocol. Real-time PCR was set up with Roche Universal Probe Library hydrolysis probes and Probes Master reagents and amplification was performed in triplicate on the LightCycler 480 (Roche, Indianapolis, IN). The primers used for genes NQO1, SOD2, GPX3 were designed using ProbeFinder online tool from Roche. Our RNA yield was 25-50 μg per sample, and 2.5 μg were used for each RT PCT reaction. We also run pairs of frozen/paraffin tumor tissue samples and all mRNAs were detected as expressed in all samples. In addition, the Spearman correlation coefficients for the various mRNA targets in the frozen-paraffin paired samples were highly statistically significant ($p<0.01$). Finally, correlation coefficients for replicates (separate preparations from the same block) were also highly statistically significant ($p<0.01$).

These quality control experiments have shown that we are able to extract high quality RNA from formalin fixed paraffin embedded ovarian cancer sections and that the detection and

quantitation obtained is highly concordant with that obtained using frozen tissue. We have now completed isolating RNA from all the sporadic patients so that we can now proceed with the paraffin validation of BRCAness profile. For this purpose we will profile tumors for the 60 genes of the BRCAness profile using the NanoString nCounter Platform. The NanoString nCounter has several advantages over standard microarray or PCR-based technologies. It is a highly reproducible and customizable system for mRNA quantification requiring only limited amounts of clinical material, even from the most challenging experimental conditions i.e. FFPE specimens. It offers the advantage over qPCR of directly measuring mRNA expression levels without enzymatic reactions and has demonstrated excellent reproducibility, reliability, detection limit and a linear dynamic range of over 500-fold.

We have now completed the Nanostring nCounter experiments for these samples and we will proceed with the data analysis in the next 6 months.

KEY RESEARCH ACCOMPLISHMENTS

1. Application of the BRCAness profile in the TCGA EOC dataset can identify tumors that have defective HR due to overexpression of certain miRNAs, in the absence of known genetic and epigenetic abnormalities of the HR pathway.
2. The HSP90 inhibitor 17-AAG enhances sensitivity of non-BRCA1/2 mutated ovarian cancer cells to DSB-inducing agents olaparib and carboplatin at very low, sublethal concentrations.
3. The mechanism for the synergistic activity of HSP90 inhibitor with PARPis and platinum is likely due to increasing DNA damage via suppressing HR.
4. High quality RNA from formalin fixed paraffin embedded ovarian cancer sections can be extracted and its detection and quantitation is highly concordant with that obtained from frozen tissue.

REPORTABLE OUTCOMES

1. The following manuscripts have been accepted/published during this funding period:

1. Copy neutral loss of heterozygosity is more frequent in older ovarian cancer patients. Pedersen BS, Konstantinopoulos PA, Spillman MA, De S. Genes Chromosomes Cancer. 2013 May 28

2. BRCA status in epithelial ovarian cancer: implications for future clinical trial design. Konstantinopoulos PA and Matulonis UA. Clinical Investigation, In Press

3. Immunohistochemical loss of BRCA1 in uterine serous cancer. Hecht JL, Konstantinopoulos PA et al, International Journal of Gynecologic Pathology, In Press

4. PRECEDENT: A Randomized Phase II Trial Comparing EC145 (Vintafolide) and Pegylated Liposomal Doxorubicin (PLD) in Combination, Versus PLD Alone, in Patients with Platinum-Resistant Ovarian Cancer. Neumann et al. JCO in Press

5. Pathologic and gene expression features of metastatic melanomas to the brain. Hamilton R, Krauze M, Romkes M, Omolo B, Konstantinopoulos P et al. Cancer. 2013 May 21. doi: 10.1002/cncr.28029

2. The following abstracts were presented in meetings (2 in national see Appendix and 1 in regional) during this funding period:

1. 2013 AACR Meeting: Chiara Battelli, Alexander Morse, Hai Hu, Elena Levantini, Gerburg Wulf, Panagiotis A. Konstantinopoulos. HSP90 inhibitor 17-allylamino-geldanamycin enhances sensitivity to double-strand DNA break-inducing agents (platinum and PARP inhibitors) in epithelial ovarian cancer (see Appendix)
2. 2013 AACR Meeting: Alexander Morse, Chiara Battelli, Hai Hu, Elena Levantini, Gerburg Wulf, Youngeun Choi, Dipanjan Chowdhury, Panagiotis A. Konstantinopoulos. Expression of miR367* confers a "BRCAness" phenotype in epithelial ovarian cancer (see Appendix)
3. 2013 DFHCC Breast Ovarian Cancer Meeting: Chiara Battelli, Alexander Morse, Elena Levantini, Gerburg Wulf, Youngeun Choi, Dipanjan Chowdhury, Panagiotis A. Konstantinopoulos. Genomic approaches to enhance sensitivity to double-strand DNA break-inducing agents in epithelial ovarian cancer

3. During this funding period I became a member of the Gynecologic Oncology Group (GOG) Experimental Medicine Committee.

4. During this funding period I was invited to serve as a reviewer for the NIH.

CONCLUSION

Our results thus far support the hypothesis that the BRCAness profile is able to track diverse molecular mechanisms that cause defective homologous recombination (HR) and that it is associated with survival in patients with sporadic disease and clinical responsiveness to platinum. Specifically, application of the BRCAness profile in the TCGA EOC dataset can identify tumors that have defective HR due to overexpression of certain miRNAs, in the absence of known genetic and epigenetic abnormalities of the HR pathway. Furthermore, the HSP90 inhibitor 17-AAG enhances sensitivity of non-BRCA1/2 mutated ovarian cancer cells to DSB-inducing agents olaparib and carboplatin at very low, sublethal concentrations. Importantly, the mechanism for this synergistic effect seems to be increasing DNA damage via suppressing HR.

Finally, high quality RNA from formalin fixed paraffin embedded ovarian cancer sections can be extracted and its detection and quantitation is highly concordant with that obtained from frozen tissue.

REFERENCES

1. Konstantinopoulos PA, Spentzos D, Karlan BY, et al. Gene expression profile of BRCAness that correlates with responsiveness to chemotherapy and with outcome in patients with epithelial ovarian cancer. J Clin Oncol 2010;28:3555-61.
2. Tchekmedyian N, Morse A, Sanisetty S, Cannistra SA, Konstantinopoulos PA. Association of a BRCAness profile with outcome and molecular aberrations involving homologous recombination in the TCGA ovarian cancer dataset. . 2012 AACR Annual Meeting; 2012; Chicago, IL; 2012.
3. Konstantinopoulos PA, Spentzos D, Fountzilas E, et al. Keap1 mutations and Nrf2 pathway activation in epithelial ovarian cancer. Cancer Res 2011;71:5081-9.
4. Lamb J, Crawford ED, Peck D, et al. The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. Science 2006;313:1929-35.

APPENDIX

The following 2 abstracts was presented in 2013 AACR Meeting:

1. HSP90 inhibitor 17-allylamino-geldanamycin enhances sensitivity to double-strand DNA break-inducing agents (platinum and PARP inhibitors) in epithelial ovarian cancer

Chiara Battelli, Alexander Morse, Hai Hu, Elena Levantini, Gerburg Wulf, Panagiotis A. Konstantinopoulos

Program of Gynecologic Medical Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts

Double-strand DNA break (DSB)-inducing agents such as platinum analogues and poly (ADP-ribose) polymerase inhibitors (PARPis) exhibit significant antitumor activity in epithelial ovarian cancer (EOC). Approximately 50% of patients with EOC harbor genetic or epigenetic alterations of the homologous recombination (HR) DNA repair pathway and are highly responsive to these agents. However, resistance to DSB-inducing agents develops in the majority of patients and represents a major limitation for successful treatment of EOC. We recently defined a gene expression profile of "BRCAness" that correlates with responsiveness to double-strand DNA break-inducing agents (platinum and PARPis) in EOC. We applied this profile to the Connectivity Map to identify candidate compounds that might be able to enhance sensitivity to these agents. This analysis showed that the top-performing compound was the heat shock protein 90 (HSP90) inhibitor 17-allylamino-geldanamycin (17-AAG) with very high connectivity scores (>0.8) across several cell lines and concentrations (permutation p value < 0.00001). We then evaluated the ability of 17-AAG to enhance sensitivity to double-strand DNA break-inducing agents in a panel of ovarian cancer cell lines. Exposure to increasing concentrations of 17-AAG (0.02-0.1uM) was associated with increased sensitivity of 36M2 and OVCAR5 cells to carboplatin in a dose response manner. Similarly, exposure to increasing concentrations of 17-AAG (0.02-0.1uM) was associated with increased sensitivity of 36M2 and OVCAR5 cells to PARPi olaparib in a dose response manner. Exposure of these cell lines to combination of 17-AAG with carboplatin induced increased phosphorylation of H2AX (gamma H2AX) - a surrogate of DNA double-strand breaks - compared to carboplatin alone, as assessed by Western blotting. In conclusion, the

HSP90 inhibitor 17-AAG enhances sensitivity of ovarian cancer cell lines to carboplatin and olaparib. The mechanism of this previously unknown, off-target effect, seems to be due to increasing DNA double strand breaks.

2. Expression of miR367* confers a "BRCAness" phenotype in epithelial ovarian cancer

Alexander Morse, Chiara Battelli, Hai Hu, Elena Levantini, Gerburg Wulf, Youngeun Choi, Dipanjan Chowdhury, Panagiotis A. Konstantinopoulos

Program of Gynecologic Medical Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts

Patients with hereditary epithelial ovarian cancer (EOC) associated with germline BRCA1/2 mutations exhibit improved outcome and high sensitivity to platinum and poly (ADP-ribose) polymerase inhibitors (PARPis) due to an underlying defect in DNA repair via homologous recombination (HR). Importantly, a subset of patients with sporadic EOCs also exhibit improved outcome and responsiveness to these cytotoxic agents - a phenotype commonly referred to as "BRCAness" - possibly due to defective HR caused by mechanisms unrelated to germline mutation in BRCA-1 or 2. Here, we report that expression of miR-367* in EOC tumors without genetic or epigenetic alterations of HR pathway genes is associated with a "BRCAness" phenotype. We accessed data from 313 high grade serous EOCs included in The Cancer Genome Atlas (TCGA) EOC dataset. For all these tumors gene expression, DNA copy number, promoter methylation and whole-exome DNA sequencing information was available. Approximately 50% of these tumors (154 of 313) had known genetic or epigenetic alterations in HR pathway genes, including somatic or germline BRCA1/2 mutations, hypermethylation of BRCA1 or RAD51C, amplification or mutation of EMSY, focal deletion or mutation of PTEN, mutation of ATM or ATR, and mutation of Fanconi anemia genes. We focused on the remaining 159 tumors that did not harbor genetic or epigenetic alterations in HR pathway and used miRNA Agilent array expression data from these tumors to correlate expression of miR-367* with outcome and platinum sensitivity. Among these 159 tumors without known genetic or epigenetic alterations of HR pathway genes, those tumors whose expression level for miR-367* was in the top tertile, were associated with improved overall survival (OS) and progression free survival (PFS), (hazard ratio for death was 0.604, p=0.033 and for progression was 0.599, p=0.025).

Similar associations with OS and PFS were identified when miR-367* expression was evaluated as a continuous variable (p=0.013 for OS and p=0.029 for PFS). Furthermore, among these 159 tumors without known genetic or epigenetic alterations of HR pathway genes, those tumors whose expression level for miR-367* was in the top tertile were more likely to be platinum sensitive (71% vs 50% respectively, two sided p=0.069). Finally, induced expression of miR-367* in ovarian cancer cell lines enhanced sensitivity to DNA double strand break-inducing agents, such as carboplatin and the PARPi olaparib in vitro. These data suggest that expression of miR-367* is associated with a "BRCAness" phenotype in EOC, i.e. improved outcome and sensitivity to platinum and PARPis. We are currently investigating whether miR-367* may interfere directly with DNA repair via HR in vitro.